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The Phosphatidylserine and Phosphatidylethanolamine Receptor CD300a Binds Dengue Virus and Enhances Infection

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ABSTRACT

Dengue virus (DENV) is the etiological agent of the major human arboviral disease. We previously demonstrated that the TIM and TAM families of phosphatidylserine (PtdSer) receptors involved in the phagocytosis of apoptotic cells mediate DENV entry into target cells. We show here that human CD300a, a recently identified phospholipid receptor, also binds directly DENV particles and enhances viral entry. CD300a facilitates infection of the four DENV serotypes, as well as of other mosquito-borne viruses such as West Nile virus and Chikungunya virus. CD300a acts as an attachment factor that enhances DENV internalization through clathrin-mediated endocytosis. CD300a recognizes predominantly phosphatidylethanolamine (PtdEth) and to a lesser extent PtdSer associated with viral particles. Mutation of residues in the IgV domain critical for phospholipid binding abrogate CD300a-mediated enhancement of DENV infection. Finally, we show that CD300a is expressed at the surface of primary macrophages and anti-CD300a polyclonal antibodies partially inhibited DENV infection of these cells. Overall, these data indicate that CD300a is a novel DENV binding receptor that recognizes PtdEth and PtdSer present on virions and enhance infection.

IMPORTANCE

Dengue disease, caused by dengue virus (DENV), has emerged as the most important mosquito-borne viral disease of humans and is a major global health concern. The molecular bases of DENV-host cell interactions during virus entry are poorly understood, hampering the discovery of new targets for antiviral intervention. We recently discovered that the TIM and TAM proteins, two receptor families involved in the phosphatidylserine (PtdSer)-dependent phagocytic removal of apoptotic cells, interact with DENV particles-associated PtdSer through a mechanism that mimics the recognition of apoptotic cells and mediate DENV infection. In this study, we show that CD300a, a novel identified phospholipid receptor, mediates DENV infection. CD300a-dependent DENV infection relies on the direct recognition of phosphatidylethanolamine and to a lesser extent PtdSer associated with viral particles. This study provides novel insights into the mechanisms that mediate DENV entry and reinforce the concept that DENV uses an apoptotic mimicry strategy for viral entry.

Dengue virus (DENV) belongs to the flavivirus genus, which encloses more than 70 enveloped positive-stranded RNA viruses, many of which are responsible for severe diseases in vertebrates (1, 2). There are four DENV serotypes (DENV-1, -2, -3, and -4) that are transmitted to humans by the mosquito vector *Aedes aegypti*. Infection with any of the DENV serotypes results in a spectrum of illness, ranging from flu-like disease (dengue fever) to more severe disease manifestations such as hemorrhagic fever and shock syndrome (3, 4). There are 390 million DENV estimated infections per year worldwide and up to 96 million dengue cases (5). No effective antiviral therapeutics or a licensed vaccine are currently available; therefore, a detailed understanding of the molecular interactions that DENV establishes with the host cell during infection is required to propose new targets for viral intervention.

DENV has a wide tropism and infects a broad range of mammalian cell types, which has hindered the identification of cellular proteins required for entry. Nonetheless, several cell surface molecules have been proposed as putative DENV entry factors and facilitate infection (reviewed in reference 6). For instance, the C-type lectin receptors—DC-SIGN on dendritic cells (DCs), the mannose receptor (MR) on macrophages, or L-SIGN on liver endothelial cells—bind glycans exposed on the DENV E protein and promote viral entry (7–11). DENV also productively infects many cell types that do not express DC-SIGN, LSIGN, or the MR, indicating that additional receptor(s) are operative.

Using a gain-of-function approach, we found that the TIM and TAM receptors also mediate DENV entry (12). The TIM and TAM molecules are two distinct families of cellular receptors that recognize phosphatidylserine (PtdSer) exposed on apoptotic cells and specialized in their elimination through phagocytosis (13–15). The TIM proteins, which include three members in humans (TIM-1, -3, and -4), interact with PtdSer exposed on apoptotic bodies through a conserved pocket in the IgV domain, termed the

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metal ion-dependent ligand-binding site (MILIBS) (14, 16, 17). TAM receptors bind indirectly to apoptotic cells through the TAM ligand Gas6/ProS, which function as bridging molecules (13). Their γ-carboxylated Gla domain interacts with PtdSer on apoptotic debris, while their LG domains bind to the Ig-like domains of TAM receptor expressed on phagocytes (13, 18). Ectopic expression of TIM or TAM receptors enhances infection by all DENV serotypes and related flaviviruses, such as West Nile virus (WNV), yellow fever virus (YFV), or Zika virus (18–20). Anti-TIM or anti-TAM antibody (Ab) block DENV infection and endogenous expression of these receptors correlates with DENV susceptibility (12). We also highlighted a crucial role of viral associated-PtdSer in DENV entry. We found that PtdSer is associated with DENV particles and is required for TIM- and TAM-mediated enhancement of DENV entry (12). By analogy with the binding of apoptotic cells to PtdSer receptors, we showed that TIM molecules interact directly with DENV-associated PtdSer through their MILIBS, whereas TAM receptors bind indirectly to viral PtdSer through their ligands Gas6 or ProS in a tripartite complex (12).

Our cDNA screen also identified CD300a as potential molecule that enhances DENV2 infection. CD300a is a phospholipid receptor that belongs to the CD300 family of paired activating/inhibitory receptors that are expressed on myeloid cells and regulate their activation (21). We describe here the role of CD300a as a novel DENV host factor that binds directly to DENV particles and enhance infection. We showed that CD300a-dependent binding and infection relies essentially on the recognition of phosphatidylethanolamine (PtdEth) and to a lesser extent PtdSer present on the surface of DENV particles.

MATERIALS AND METHODS

Cell lines and virus strains. HEK293T cells, HeLa cells (a gift from L. Pelkmans, University of Zurich, Zurich, Switzerland), and Vero cells were maintained in Dulbecco modified Eagle medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and L-glutamine (Life Technologies). HEK293T and Vero cells were purchased from the American Type Culture Collection (ATCC). HEK293T and HeLa cells stably expressing CD300a wild type (WT) or mutants were generated by transduction using the pTRIP lentiviral vectors as previously described (12). Cell populations with high surface expression of CD300a were sorted using a BD FACSAria II cell sorter with the FACSDiva 6.1.2 software (Becton Dickinson). DENV serotype 1 (DENV1) TVP5175, DENV2 JAM (Jamaica), DENV2 NGC (New Guinea C), DENV3 (PAH-881), and DENV4 (1036), as well as WNV lineage I (IS-98-ST1), YFV Asibi, and Chikungunya CHIK21 strain (CHIKV) viruses were propagated in mosquito (*Aedes pseudoscutellaris*) AP61 cell monolayers (a gift from Philippe Despres, Institute Pasteur) with limited cell passages. Viruses were purified through a 20% sucrose cushion by ultracentrifugation at 80,000 × *g* for 2 h at 4°C. Pellets were resuspended in TNE1X (pH 7.4; 50 mM Tris, 100 mM NaCl, 0.5 mM EDTA), divided into aliquots, and stored at −80°C. Titers were determined on Vero cells by flow cytometry analysis and expressed as flow cytometry infectious units (FIU). Herpes simplex virus 1(F) [HSV-1(F)] was propagated, and the titer was determined on Vero cells as described previously (12).

Monocytes, MDMs, and mast cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from normal donors over a Ficoll-Paque (GE Healthcare) according to the manufacturer's instructions. Monocytes were purified from PBMCs by negative selection (depletion of nonmonocytes) using a Monocyte Isolation Kit II (Miltenyi Biotech) according to the manufacturer's recommendations. Purified monocytes were either used for infection assay or cultured in RPMI 1640 supplemented with granulocyte-macrophage colony-stimulating factor (2 ng/ml) and macrophage colony-stimulating factor (20 ng/ml) for 7 days to generate monocyte-derived macrophages (MDMs). Mast cells (a gift from Michel Arock) were generated as previously described (22).

Ethics statement. Blood from healthy adult donors was provided by the Etablissement Français du Sang (EFS), Paris, France, within the framework of a bilateral agreement between EFS and Hôpital Saint-Louis. All samples were collected in accordance with EU standards and national laws and were anonymized.

Reagents. The recombinant human Ig1-Fc, NKG2D-Fc, human and mouse CD300a-Fc, and DC-SIGN-Fc were purchased from R&D Systems. The CD300c-Fc was from J. Kitaura. Antibodies to the human CD300a/c included mouse IgG1 MAb, clone MEM260 (Abcys), and goat IgG Ab AF2640 (both from R&D Systems). The mouse CD300a was detected with the rat IgG2a MAb clone 172224 (R&D Systems). CD300a- and CD300c-specific antibodies were rat monoclonal Ab (MAb) 6-2a and mouse 1E7D, respectively, provided by J. Kitaura. Clathrin heavy-chain and β-tubulin rabbit pAb were from Abcam. The DENV antibodies were mouse MAbs: anti-DENV NS1 protein (provided by Michael Diamond [Saint Louis, MO]), anti-DENV prM 2H2, anti-panflavivirus E protein 4G2, and anti-WNV E proteins E16 and anti-YFV E proteins 2D12 MAbs. Infection by HSV-1 was detected by an anti ICP4 MAb (Santa Cruz Biotechnologies). The horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-human IgG was from Dako Cytomation. The Alexa Fluor 488-conjugated goat anti-mouse IgG were from Jackson ImmunoResearch. annexin V and duramycin were both from Sigma (Lyon, France).

cDNA library screening and plasmid constructs. Details of the arrayed library and screening method are described elsewhere (12). The human *CD300a* open reading frame (ORF) was amplified from the cDNA library, and the mouse *CD300a* and human *CD300c* ORFs were amplified from cDNA provided by J. Kitaura (Tokyo, Japan). These ORFs were cloned as BamHI/XhoI digested fragments into the likewise-digested pTRIP vector. Mutants of CD300a were generated using QuikChange site-directed mutagenesis (Agilent). The Eps15Δ95-295 green fluorescent protein (GFP) and GFP control constructs were described elsewhere (23).

Flow cytometry analysis. Cells were detached with 2.5 mM EDTA in phosphate-buffered saline (PBS) and incubated with primary Ab (5 μg/ml) in 100 μl of PBS with 0.02% NaN_3 and 5% FBS for 1 h at 4°C. After the primary staining, the cells were washed and incubated with the appropriate Alexa 488-conjugated secondary antibody (Jackson ImmunoResearch) for 30 min at 4°C. For infection assay analysis, cells were detached with trypsin/EDTA (Life Technologies) and fixed with PBS plus 2% (vol/vol) paraformaldehyde (PFA) for 15 min at 4°C. Intracellular viral antigens were stained with the appropriate mouse MAbs, followed by a secondary staining with an Alexa 488-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) in PBS with 0.02% NaN_3, 5% FBS, and 0.5% (wt/vol) saponin. Acquisition was performed on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson), and data were analyzed by using FlowJo software (Tree Star, Olten, Switzerland).

Internalization assay. Cells were cultured on Lab-Tek II-CC^2 chamber slide (Nunc, Roskilde, Denmark) for 2 days. Prechilled cells were incubated with viral particles on ice for 1 h. Cells were washed twice with cold PBS to remove unbound particles, and internalization was initiated by adding prewarmed medium and shifting the cells at 37°C for 45 min. The cells were then incubated on ice, washed with cold PBS, and fixed in 4% PFA (vol/vol) for 20 min at 4°C. The DENV particles were stained with the 4G2 anti-E MAb antibody, followed by a secondary staining with Alexa 488-conjugated goat anti-mouse IgG. Immunostaining was performed in dual conditions, in the continuous presence of 0.1% saponin (permeabilized) or without saponin (unpermeabilized). Slides were mounted with ProLong Gold antifade reagent containing DAPI (4′,6′-diamidino-2-phenylindole) for nuclei staining (Life Technologies).

Virus pulldown and ELISA. Virus pulldown experiments were performed as previously described (12). All enzyme-linked immunosorbent assays (ELISAs) were performed on Maxisorp 96-well plates (Nunc). To detect direct interactions between CD300a-Fc and DENV, plates were coated overnight in duplicates with DENV particles (10^6 FIU) diluted in

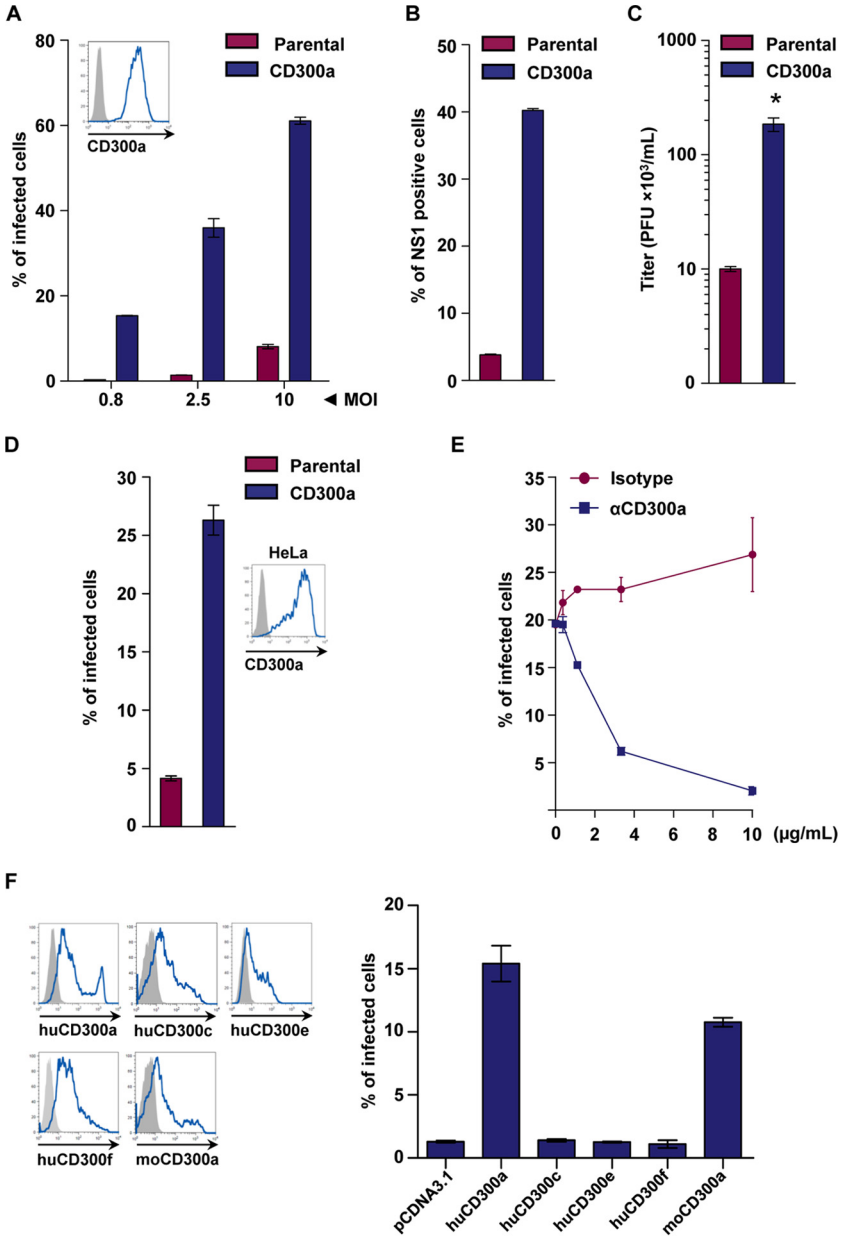

FIG 1 Ectopic expression of CD300a enhances DENV2 infection. In panels A and D, the inset displays the cell surface expression analysis by flow cytometry of CD300a in parental (gray shading) and transduced cells (blue line). (A) Parental and 293T cells expressing CD300a receptor were challenged with DENV2 JAM at the indicated MOIs. The percentages of infected cells were quantified 24 h later by flow cytometry using the anti-prM 2H2 MAb. (B) Parental and 293T cells expressing CD300a were challenged with DENV2 JAM (MOI 2), and the percentages of infected cells were quantified 24 h later by flow cytometry with an antibody directed against the nonstructural NS1 viral protein. (C) Parental and CD300a-expressing cells were incubated 3 h with DENV2 JAM (MOI = 10). Supernatants were collected 48 h postinfection, and virus titers were determined by plaque assays on C6/36 cells and are expressed as PFU/ml. Significance was calculated by using a two-sample Student *t* test (*, $P < 0.05$). (D) Parental and CD300a-expressing HeLa cells were infected with DENV2 JAM, and the percentages of infected cells were quantified 24 h later with the 2H2 MAb. (E) CD300a-expressing and parental 293T cells were preincubated for 30 min with increased concentrations of a polyclonal goat anti-CD300a or a control IgG. The cells were infected DENV2 JAM (MOI = 2) in the continuous presence of the antibodies, and infection was quantified 24 h later by flow cytometry with 2H2 MAb. (F) 293T cells were transfected with plasmids for the human CD300a, CD300c, CD300e, and CD300f, the mouse CD300a, or pCDNA3.1 backbone vector as a control. (Left panel) The cell surface expression levels of CD300 molecules were analyzed at the time of infection by flow cytometry after staining with the respective antibodies for each antigen (blue line) and the matched control isotypes (gray shading). (Right panel) Cells were infected with DENV2 JAM (MOI = 10), and infection was assessed 24 h later with the 2H2 MAb. In panels A to F, the data shown are means ± the SD from three independent experiments performed in duplicate.

PBS at 4°C. For the phospholipid binding assay, plates were coated in duplicates with 3-*sn*-phosphatidylcholine, 3-*sn*-phosphatidylethanolamine, or 3-*sn*-phosphatidylserine (10 μg/well; Sigma) diluted in ethanol, and allowed to air dry at room temperature. Wells were saturated with 1× Tris-buffered saline (TBS), 10 mM $CaCl_2$, and 2% bovine serum albumin (BSA) for 2 h at 37°C before incubation with 100 μl of Fc-chimera (2 μg/ml) in 1× TBS, 10 mM $CaCl_2$, and 0.5% Tween 20. The wells were extensively washed, and bound Fc-chimeras were detected with HRP-

conjugated anti-human IgG antibody. For competitive binding studies, annexin V or Duramycin were preincubated in the wells with coated viruses, whereas EDTA or antibodies were preincubated with the Fc-fusion proteins in TBS 1×, 10 mM $CaCl_2$, and 0.5% Tween 20.

Cell binding assay. Cells were detached with 2.5 mM EDTA in PBS and incubated with 100 U of heparin (Sigma) for 30 min at 4°C. Cells (4×10^5) were then incubated with DENV2 JAM (multiplicity of infection [MOI] = 100) for 90 min at 4°C in binding buffer (DMEM, 0.05% NaN_3) containing 2% BSA. The cells were washed twice with cold binding buffer, once with serum-free cold DMEM, and fixed with 2% PBS-PFA at 4°C for 20 min. Cell surface-absorbed DENV particles were stained with the anti-panflavivirus envelope 4G2 antibody (1 μg/ml), followed by secondary staining with Alexa 488-conjugated goat anti-mouse IgG, and then analyzed by flow cytometry.

Phagocytosis experiments. Murine thymocytes were incubated with 20 μM dexamethasone (Sigma) in RPMI 1640 for 3 h at 37°C to induce apoptosis. After treatment, apoptotic thymocytes were washed twice and labeled with the pH-sensitive dye pHrodo-Red (Life Technologies) at 100 ng/ml for 30 min at room temperature. The cells were then washed with PBS containing 10% FBS and then resuspended in cold RPMI 1640 medium without serum at 10^6 cells/ml to be used for engulfment. 293T cells expressing TIM1 or CD300a were grown in 24-well plates and counted to estimate the ratio of apoptotic thymocytes versus receptor-expressing cells (10:1). The cells were then incubated with the labeled apoptotic thymocytes at 4°C for 20 min to allow the binding but not the engulfment of apoptotic cells. Apoptotic cell uptake was initiated by shifting the cells to 37°C for 2 h. The cells were washed twice with PBS-EDTA to remove uninternalized apoptotic cells, detached with 0.05% trypsin-EDTA (Gibco), and resuspended in PBS supplemented with 2% FBS (pH 9). The percentage of cells that engulfed apoptotic thymocytes was determined by flow cytometry detection of the fluorescence emitted by the pHrodo dye in acidic environment. As a control, the fluorescence of the cells kept at 4°C was also assessed by flow cytometry.

RNA interference. HeLa cells expressing CD300a were transfected according to the Lipofectamine RNAiMAX protocol (Life Technologies) with 10 nM ON-TARGETplus Smartpool small interfering RNAs (siRNAs) targeted for the clathrin heavy chain (CHC; L-004001-01) or with nonspecific Smartpool control siRNA (NT; D-001810-10) (both from Dharmacon). After 72 h, the cells were infected with DENV2 JAM, and infected cells percentages were quantified at 24 h postinfection by flow cytometry.

RNA purification, cDNA synthesis, and RT-qPCR. Total RNA was extracted from infected cells by using an RNeasy Plus minikit (Qiagen) and stored at −80°C. cDNA was synthesized from 500 ng of total isolated RNA by random priming-reverse transcription (RT) with the SuperScript VILO cDNA synthesis kit (Life Technologies). Real-time quantitative PCR (qPCR) was performed using a Fast SYBR Green Master Mix kit (Life Technologies) on an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies). The primers for viral RNA quantification were designed with PrimerExpress software V2.0 (Life Technologies) within the conserved region of the capsid gene of DENV2 JAM (forward primer, 5′-TTCTCACTTGGAATGCTGCAA-3′; reverse primer, 5′-GCCACAAG GGCCATGAAC-3′), and QuantiTect primers for GAPDH were purchased from Qiagen. The relative expression quantification was performed based on the comparative threshold cycle (C_T) method, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an endogenous reference control.

Statistical analyses. Graphical representation and statistical analyses were performed using Prism5 software (GraphPad Software). Unless otherwise stated, the results are expressed as means ± the standard deviations (SD) from three independent experiments performed in duplicate, and differences were tested for statistical significance using a paired two-tailed *t* test.

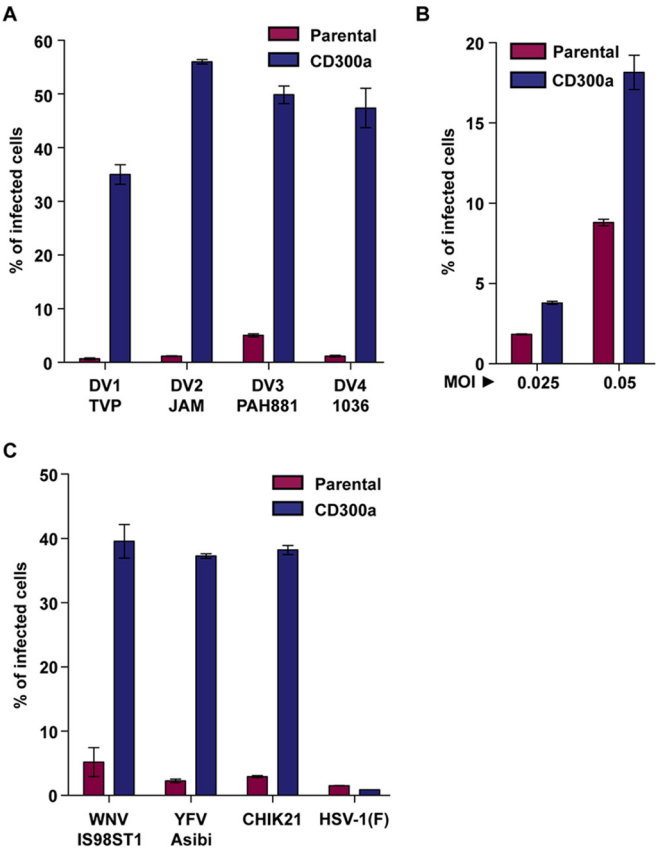

FIG 2 CD300a is exploited by the four DENV serotypes and other mosquito-borne viruses. (A to C) CD300a-expressing and parental 293T cells were infected with DENV1 TVP5175 (MOI = 20), DENV2 JAM (MOI = 10), DENV3 PAH881 (MOI = 10), or DENV4 1036 (MOI = 20) (A), DV2 NGC (MOI = 0.5) (B), or IS98ST1 (MOI = 0.005), YFV Asibi (MOI = 2), Chikungunya CHIK21 (MOI = 10), and HSV-1(F) (MOI = 0.5) (C) virus strains. The percentages of infected cells were assessed 24 h postinfection by flow cytometry with respective antiviral protein antibodies. The data are means ± the SD of three independent experiments performed in duplicate.

RESULTS AND DISCUSSION

CD300a enhances DENV infection. We previously performed a gain-of-function cDNA screen to identify cellular receptors that mediate DENV infection (12). In addition to the TIM and TAM receptor families (12), this strategy identified CD300a as a molecule that enhances DENV2 JAM infection. CD300a belongs to the CD300 family of paired activating/inhibitory receptors which are expressed on myeloid cells and regulate their activation (21). To further characterize the role of CD300a during DENV infection, we generated HEK293T cells expressing CD300a (293T-CD300a cells) and challenged them with DENV2 JAM at different multiplicities of infection (MOIs). Viral infection was quantified 48 h later by flow cytometry, using the 2H2 MAb that recognizes the DENV prM protein. Compared to parental cells, we observed a massive increase of DENV infection in CD300a expressing cells (Fig. 1A). Similar results were obtained when measuring newly synthesized NS1, a viral structural protein produced only during active replication (Fig. 1B). Titration of the supernatants showed that 293T-CD300a cells released 20 times more infectious particles than parental cells (Fig. 1C). CD300a-mediated enhancement of DENV2 infection was also observed when HeLa cells were used as recipient (Fig. 1D).

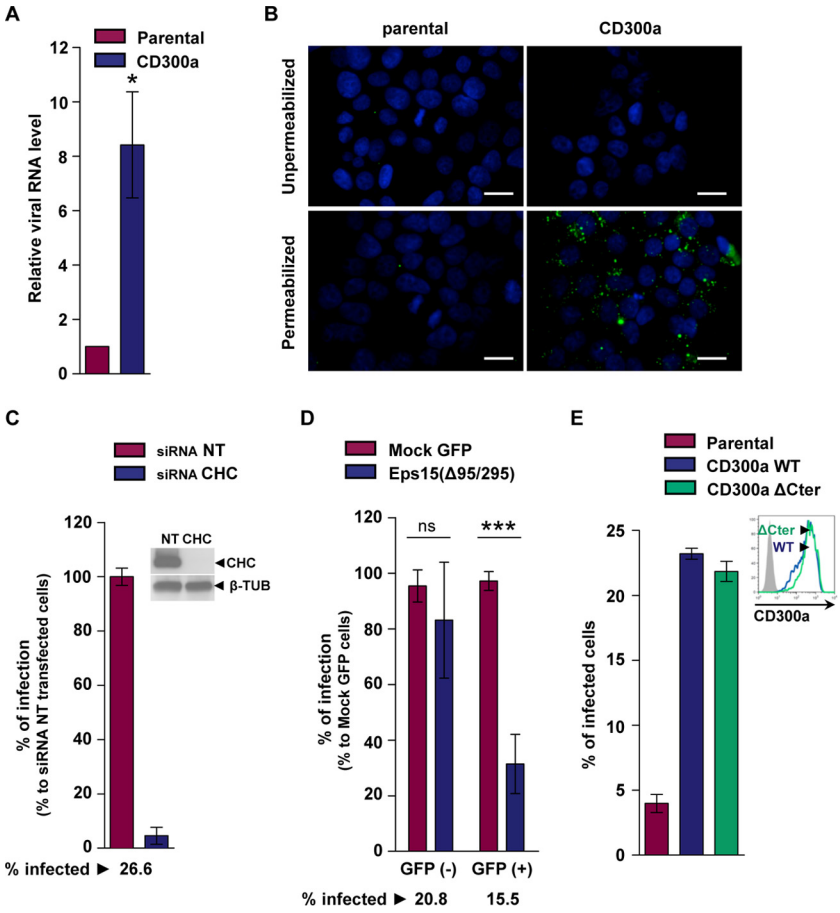

FIG 3 CD300a facilitates DENV endocytosis through the clathrin-dependent pathway. (A) DENV2 JAM viral particles (MOI = 10) were prebound to CD300a-expressing and parental 293T cells for 1 h at 4°C. Entry was initiated by shifting cells to 37°C. After 2 h, the cells were treated with proteinase K (1 μg/ml) at 4°C for 45 min to remove uninternalized particles, and the total cellular RNA was extracted. Internalized viral RNA levels were determined by real-time qPCR with human GAPDH as an endogenous control. The results are expressed as the fold difference using expression in 293T infected cells as the calibrator value. The results shown are representative of three independent experiments performed in triplicates. Significance was calculated by using a two-sample Student *t* test (*, $P < 0.05$). (B) DENV2-JAM particles (MOI = 100) were absorbed on CD300a-expressing and parental 293T cells for 1 h at 4°C, and cells were extensively washed to remove unbound particles. Internalization was initiated by shifting the cells to 37°C, and after 45 min the cells were fixed with 4% PFA. Cells were labeled with DAPI (blue) and anti-DENV E protein 4G2 (green) to detect virus uptake in unpermeabilized and permeabilized cells. Magnification, ×40. Scale bar, 20 μm. The results shown are representative of three independent experiments. (C) CD300a-expressing HeLa cells were transfected with an siRNA pool targeting for the clathrin heavy chain (siCHC) or a nontargeting negative-control siRNA pool (siNT). At 72 h posttransfection, the cells were infected with DENV2 JAM (MOI = 10). Silencing was assessed by immunoblotting at the time of infection (inset). Infection level was assessed 24 h later by flow cytometry with the 2H2 anti-prM MAb. The percent infection was normalized to the percent infection in siNT-transfected cells. The number underneath the figure indicates the percentage of infected cells in siNT-transfected cells. (D) CD300a-expressing 293T cells were transiently transfected with plasmids expressing EGFP-Eps15 negative dominant (Δ95/295) mutant or the GFP empty plasmid as a control (Mock GFP). Cells were challenged with DENV2-JAM (MOI = 10), and the infection level was assessed 24 h later by flow cytometry with the 2H2 anti-prM MAb. For each sample, the percentage of infected cells was quantified in cell populations that were negative for GFP-fusion protein (GFP−) and in cell populations containing high levels of the protein (GFP+). The percent infection was normalized to the percentage of infection in control-transfected cells. Numbers underneath the figure indicate the percentages of infected cells in control-transfected cells. Significance was calculated by comparing the percentage of infection in Esp15(Δ95/295)-transfected cells to the percentage of infection in mock-transfected cells using a two-sample Student *t* test (ns, nonsignificant; ***, $P < 0.0001$). (E) 293T cells stably expressing the WT CD300a or a CD300a molecule truncated for the cytoplasmic domain (CD300aΔC_Ter) were challenged with DENV2 JAM at an MOI of 2. The inset displays wild-type (WT) and mutant CD300a cell surface expression. Infection level was assessed 24 h later by flow cytometry with the 2H2 anti-prM MAb. (C to E) The data shown are means ± the SD of three independent experiments performed in duplicate.

We then examined whether polyclonal Ab recognizing human CD300a blocked infection. Preincubation of CD300a-expressing cell with a polyclonal anti-CD300a Ab, but not a control Ab, inhibited DENV infection in a dose-dependent manner, reducing more than 80% the infection at the highest concentration (Fig. 1E). The CD300 family includes seven related proteins in humans (21). To examine whether other members of the CD300 family mediate DENV infection, we transfected plasmids encoding human (hu) CD300a, CD300c, CD300e, and CD300f, as well as the mouse (mo) CD300a ortholog in 293T cells. Flow cytometry analysis showed that the molecules were well expressed (Fig. 1F). hu or mo CD300a enhanced DENV infection, whereas expression CD300c, CD300e, or CD300f were ineffective (Fig. 1F).

CD300a is exploited by the four DENV serotypes and by other mosquito-borne viruses. We next assessed the effect of CD300a on other DENV serotypes. We challenged 293T-CD300a

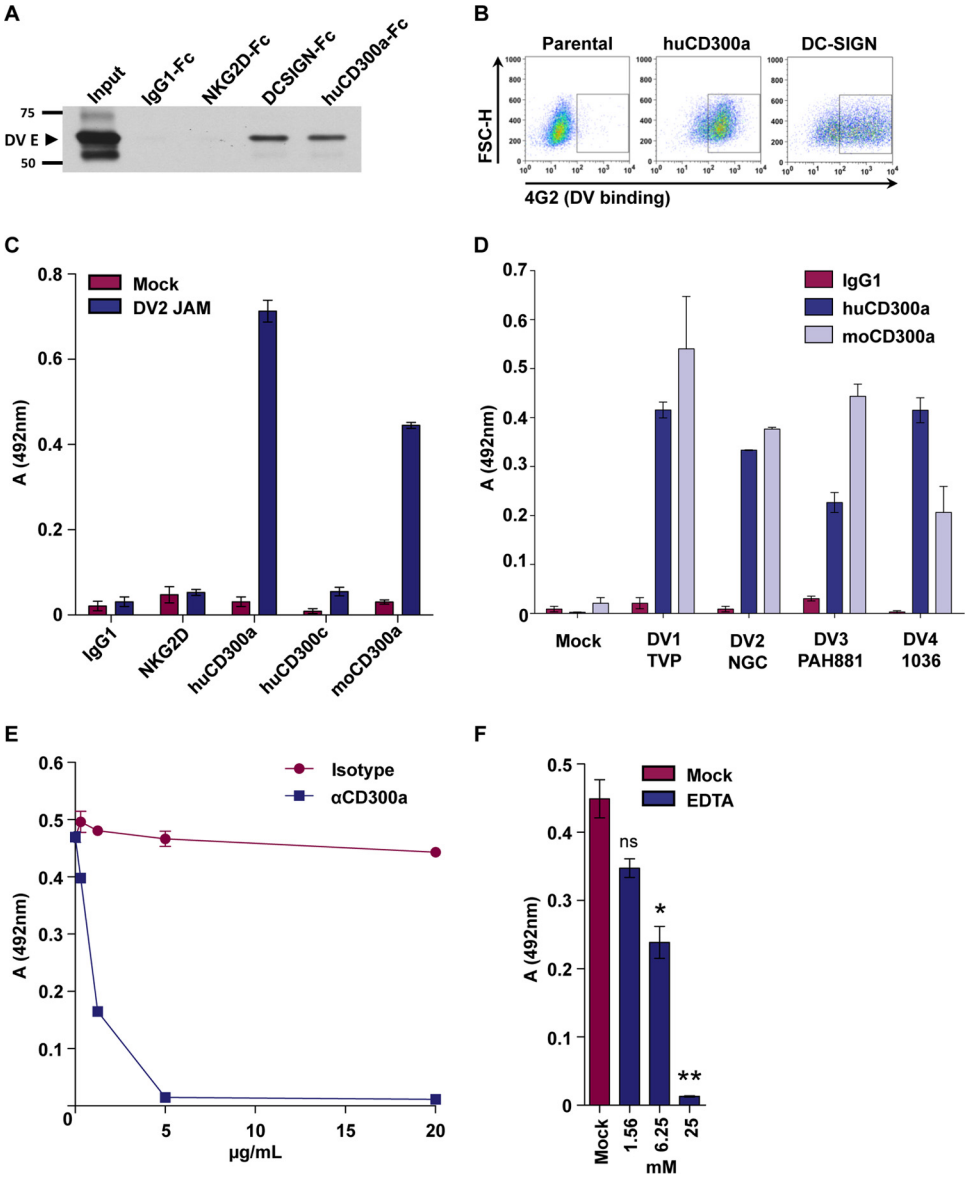

FIG 4 CD300a directly interacts with DENV. (A) Western blot analysis of DENV2 JAM particles pulled down with human CD300a. DENV2 JAM particle were preincubated overnight at 4°C with IgG1-Fc, NKG2D-Fc, CD300a-Fc, or DC-SIGN-Fc prior to incubation with protein A-Sepharose beads. Pulled-down virus was detected by immunoblotting with the 4G2 anti-E MAb. (B) Parental, CD300a, or DC-SIGN-expressing 293T cells were incubated with DENV2 JAM (MOI = 100) for 1 h at 4°C. Cell surface-bound viruses were stained with the 4G2 anti-E MAb and detected by flow cytometry. The results shown in panels A and B are representative of two independent experiments. (C) DENV2 JAM particles (10^6 FIU) were coated on 96-well plate and incubated for 1 h with IgG1-Fc, NKG2D-Fc, and human or mouse CD300a-Fc or CD300c-Fc (2 μg/ml). Bound Fc-chimera molecules were detected with an HRP-conjugated anti-human IgG (1 μg/ml). (D) DENV1 TVP5175, DENV2 NGC, DENV3 PAH881, and DENV4 1036 (10^6 FIU) particles were coated on 96-well plate and incubated for 1 h with IgG1-Fc or human or mouse CD300a-Fc (2 μg/ml). (E and F) DENV2 JAM particles (10^6 FIU) were coated onto 96-well plate. (E) Increasing concentrations of a goat anti-CD300a or a normal goat IgG were preincubated for 30 min with CD300a-Fc prior to incubation with immobilized DENV2 JAM particles. (F) The indicated concentrations of EDTA (1.56 to 25 mM) or mock treatments were mixed with human CD300a-Fc before incubation with immobilized DENV2 JAM particles. The significance was calculated by comparing Fc-CD300a binding without EDTA to that with the indicated concentration of EDTA using a two-sample Student *t* test (ns, nonsignificant; *, $P < 0.05$; **, $P < 0.001$). In panels C to F, the data shown are means ± the SD of three independent experiments performed in duplicate.

and control cells with DENV1, DENV3, and DENV4 primary isolates. CD300a enhanced infection with these viruses by 10- to 40-fold (Fig. 2A). CD300a moderately enhanced (3-fold) infection by the laboratory adapted DENV2 NGC strain (Fig. 2B). Furthermore, CD300a also mediated infection by the mosquito-borne viruses WNV, YFV, or Chikungunya virus (CHIKV) but had no effect on HSV-1 (Fig. 2C). Altogether, these results indicate that the ectopic expression of CD300a enhances the infection by the four DENV serotypes and by the mosquito-borne viruses YFV, WNV, and CHIKV.

CD300a enhances DENV internalization. We then investigated whether CD300a mediates virus internalization. 293T-CD300a and control cells were exposed to DENV2 JAM for 2 h at 37°C and treated with proteinase K to eliminate cell surface virus

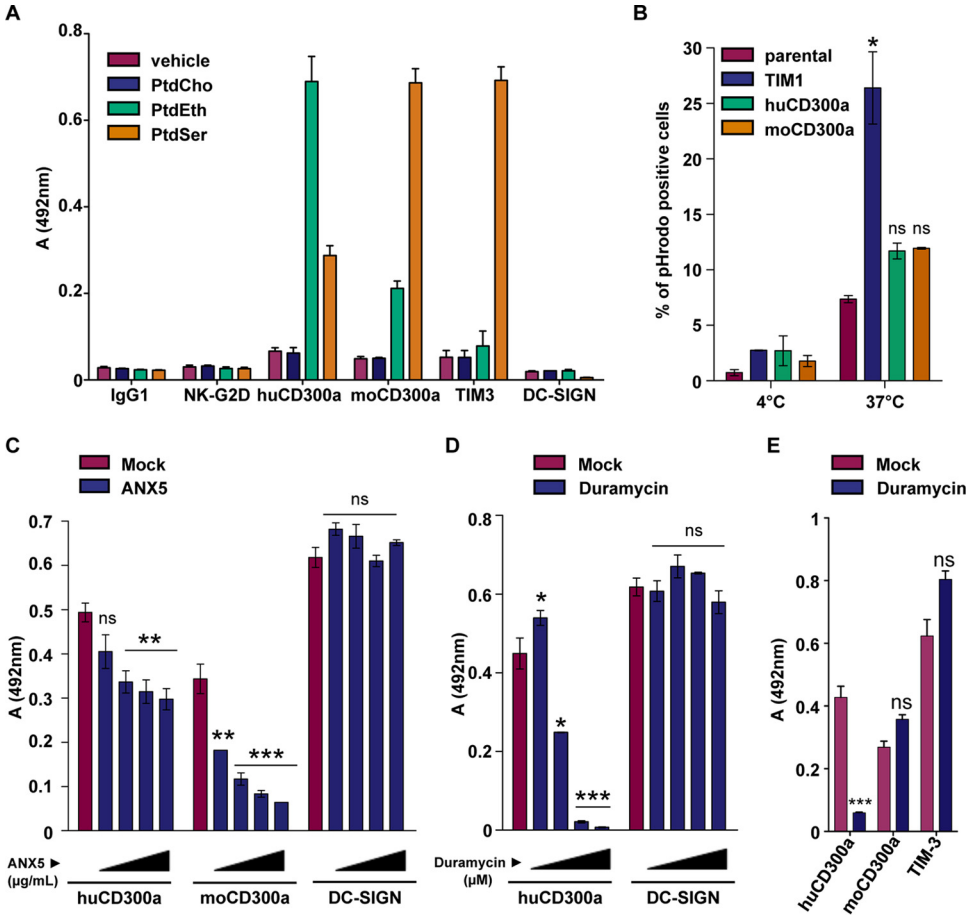

FIG 5 Human CD300a interacts with PtdEth and PtdSer associated with DENV particles. (A) 3-*sn*-PtdCho, 3-*sn*-PtdEth, or 3-*sn*-PtdSer was coated onro 96-well plates in the presence of ethanol (vehicle). Wells were incubated with human IgG1-Fc, NK-G2D-Fc, TIM3-Fc, DC-SIGN-Fc, and human or mouse CD300a-Fc (2 μg/ml). The data shown are means ± the SD of two independent experiments performed in duplicate. (B) 293T cells stably expressing TIM1 or human or mouse CD300a were incubated for 20 min at 4°C with pHrodo-labeled mouse thymocytes (1:10 ratio). Efferocytosis was initiated by shifting the cells to 37°C, and the percentages of cells that engulfed apoptotic thymocytes were detected by flow cytometry after 2 h of incubation. Cells kept at 4°C were used as a control for background fluorescence. These experiments were repeated three times in duplicate, and the data shown are means ± the SD from one representative experiment. Significance was calculated by comparison with the percentage of fluorescent parental cells using a two-sample Student *t* test (ns, nonsignificant; *, $P < 0.05$). (C and D) Immobilized DENV2 JAM particles (10^6 FIU) were preincubated for 1 h with increasing concentrations of the PtdSer-specific ligand annexin V (0.31 to 20 μg/ml) (C) or the PtdEth-specific ligand Duramycin (0.625 to 5 μM) (D) prior to the addition of the human or mouse CD300a-Fc or DC-SIGN-Fc (2 μg/ml). The data shown are means ± the SD from three independent experiments performed in duplicate. Significance was calculated by comparing binding without blocking reagents to the binding with the indicated concentration of blocking reagents using a one-way analysis of variance statistical test (ns, nonsignificant; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). (E) Immobilized DENV2 JAM particles (10^6 FIU) were preincubated for 1 h with Duramycin (5 μM) prior to addition of the human CD300a-FC or the mouse CD300a-Fc or TIM-3-Fc (2 μg/ml). The data shown are means ± the SD from two independent experiments performed in duplicate. Significance was calculated by comparing binding in presence or absence of the Duramycin using a two-sample Student *t* test (ns, nonsignificant; ***, $P < 0.0001$).

(23). Viral RNA levels were then quantified by qPCR (12). CD300a allowed an average 8-fold increase of DENV viral RNA uptake (Fig. 3A). We then incubated 293T-CD300a and control cells with DENV2 particles at 4°C for 1 h and shifted the cells to 37°C for 30 min in order to initiate endocytosis. The intracellular uptake of viruses was analyzed by fluorescence microscopy using the anti-DENV E MAb 4G2 in cells that were permeabilized, or not, with saponin as previously described (12) (Fig. 3B). We observed an intense intracellular accumulation of DENV E protein in saponin-permeabilized cells, only in the presence of CD300a (Fig. 3B). Only a few virus particles were detected in cells not treated with saponin, indicating that the majority of the viral particles were internalized after CD300a ligation.

Clathrin-mediated endocytosis (CME) is the major pH-dependent route used by DENV to enter target cells (24, 25) To investigate whether CD300a-mediated enhancement of DENV infection required a functional clathrin-dependent pathway, we silenced with siRNAs the clathrin heavy chain (CHC), one of the major components of the clathrin triskelion. The effective knockdown of CHC was verified by Western blotting (Fig. 3C), and the cells were then infected with DENV2 JAM. Silencing of CHC drastically inhibited CD300a-mediated DENV infection compared to cells transfected with a control siRNA (Fig. 3C). Eps15 regulates CME by recruiting the adaptor protein complex AP2. Deletion of the second and third Eps15 homology (EH) domains in Eps15 results in a dominant-negative protein (Eps15Δ95-295) that prevents CME (26). To determine whether CD300a-mediated entry depends on Eps15, HEK293T cells transfected with Eps15Δ95-

295-GFP or GFP plasmids were infected with DENV2 JAM (Fig. 3D). For each sample, viral infection was quantified in GFP-positive and -negative cell populations. Eps15Δ95-295 GFP inhibited infection by 70% compared to control GFP-positive cells, while the GFP-negative populations in both transfections presented similar levels of infection. Thus, CD300a-mediated entry of DENV proceeds mainly through CME. We next generated HEK293T cells stably expressing a truncated version of CD300a lacking the cytoplasmic domain (CD300aΔ$C_T$er). The human CD300a cytoplasmic domain contains four tyrosine residues that are involved in three classical and one nonclassical ITIM domain and are required for CD300a signaling (27). CD300aΔ$C_T$er mediated DENV2 JAM enhancement of infection as efficiently as its WT counterpart (Fig. 3E), indicating that the cytosolic region of CD300a is dispensable for promoting DENV infection. Collectively, our results indicate that CD300a enhances clathrin-mediated entry of DENV through a mechanism that is independent of its cytoplasmic tail. These data suggest that CD300a may act mainly as a DENV attachment receptor, whereas virus internalization is mediated by additional receptor(s) that is likely poorly expressed on the surfaces of 293T cells.

CD300a directly interacts with DENV particles. To determine whether DENV directly binds CD300a, we first conducted pulldown assays with soluble CD300a-Fc or as control IgG1, NKG2D-Fc, or DC-SIGN-Fc molecules. We incubated DENV particles with chimeric Fc proteins and protein G-agarose beads and analyzed the precipitated viruses by Western blotting with the anti-DENV E MAb 4G2. We detected a DENV-E protein band only with CD300a-Fc and our positive control DC-SIGN-Fc, but not with NKG2D- or IgG1-Fc (Fig. 4A). To assess whether DENV binds CD300a at the plasma membrane, we conducted a virus attachment assay with parental, CD300a or DC-SIGN expressing HEK293T cells (Fig. 4B). Cells were incubated with DENV particles at 4°C, and bound virions were detected with the anti-DENV E MAb DENV. A positive surface staining for DENV was obtained with cells expressing either CD300a or DC-SIGN (Fig. 4B). A direct DENV-CD300a interaction was also validated by ELISA. DENV particles were coated on plate wells prior incubation with soluble CD300a Fc molecules (Fig. 4C). Both human and mouse CD300a-Fc, but not CD300c-Fc molecules, bound to DENV2 (Fig. 4C), as well as to DENV1, DENV3, and DENV4 particles (Fig. 4D). The binding of DENV to CD300a-Fc was inhibited in a dose-dependent manner by the polyclonal anti-CD300a Ab but not by control Ig (Fig. 4E). EDTA also inhibited binding (Fig. 4F), suggesting that it is Ca^{2+} dependent. Together, these results show that CD300a mediates virus attachment to target cells and directly binds to virions.

Human CD300a interacts mainly with PtdEth associated with DENV particles. Although CD300a was originally identified as PtdSer receptor (28), the ligand specificity of CD300a is still a matter of debate since it has been proposed that hu CD300a is a phospholipid receptor that recognizes both PtdSer and PtdEth expressed on apoptotic cell membranes (29). To verify the CD300a ligand specificity, we set up an ELISA by presenting immobilized phosphatidylcholine (PtdCho), PtdEth, or PtdSer to soluble CD300a-Fc molecules (Fig. 5A). We showed that hu CD300a-Fc bound mainly to PtdEth and recognizes modestly PtdSer. Interestingly, moCD300a-Fc bound mainly PtdSer, while TIM-3 (which was used as a positive control) selectively recognized PtdSer. We next sought to determine whether hu and mo

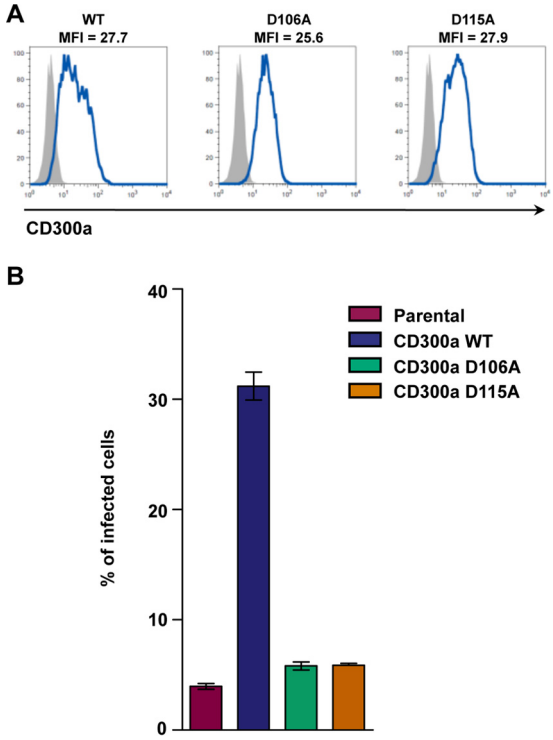

FIG 6 Mutation of aspartic acid residues crucial for PtdSer and PtEth binding abolishes CD300a-mediated DENV infection. (A) 293T cells were transduced with WT CD300a or D106A or D115A mutants, and cell surface expression of the molecules was analyzed by flow cytometry. The mean fluorescence intensities (MFI) are reported. (B) Transduced cells were challenged with DENV2 JAM (MOI = 2), and the infection level was assessed 24 h later by flow cytometry using the 2H2 anti-prM MAb. The data are means ± the SD of two independent experiments performed in duplicate.

CD300a were able to promote the phagocytosis of apoptotic mouse thymocytes. For this, apoptotic thymocytes labeled with the pH-sensitive fluorochrome pHrodo (a dye that emits a red fluorescence only at acidic pH) were incubated for 2 h at 37°C with parental, hu CD300a, mo CD300a HEK293T cells or as positive control with TIM-1 HEK293T cells. Phagocytosis was quantified by flow cytometry as the percentage of pHrodo-positive cells. In contrast to TIM-1, we found that neither hu CD300a nor mo CD300a significantly enhanced the capacity of HEK293T cells to engulf apoptotic cells (Fig. 5B). Together, these results corroborate previous reports indicating that CD300a recognizes PtdSer and PtdEth on the surfaces of apoptotic cells and does not mediate their phagocytosis (29, 30).

To investigate the respective roles of PtdSer and PtdEth in DENV binding, ELISA plates were coated with DENV2 JAM and incubated with annexin V or duramycin before the addition of hu CD300a-Fc, mo CD300a-Fc, or as control DC-SIGN-Fc molecules. Annexin V (ANX5) recognizes PtdSer, while duramycin, a 19-amino-acid peptide of the L antibiotic family, binds to PtdEth (29, 31). Annexin V modestly inhibited the binding of hu CD300a-Fc (33%) to DENV particles, while duramycin inhibited this interaction by up to 90% (Fig. 5C). mo CD300a binding to DENV was blocked up to 80% by annexin V (Fig. 5C). The inhibitory effect exerted by ANX5 and duramycin was not due to a virucidal effect of these compounds, since DC-SIGN binding to virions remained unaltered (Fig. 5D). In addition, duramycin did

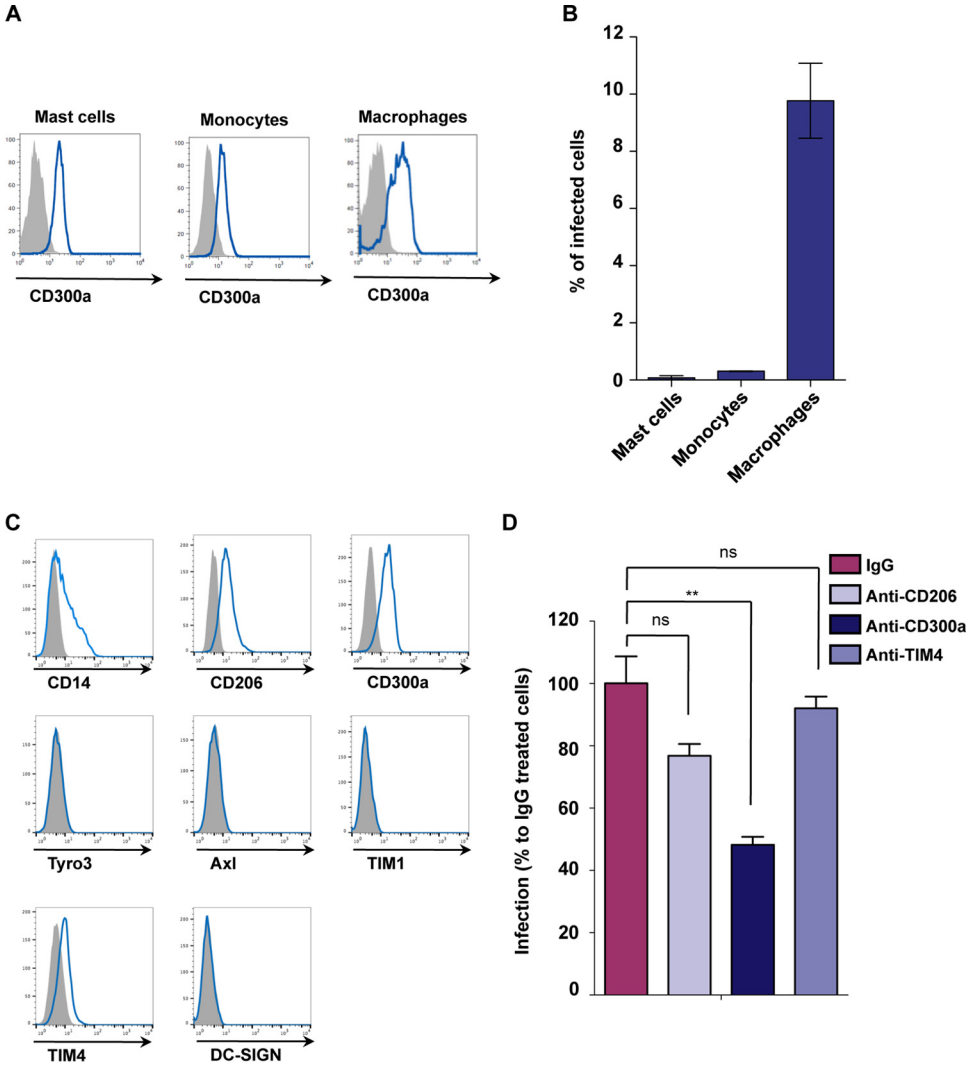

FIG 7 CD300a neutralizing antibodies partially block DENV infection in MDMs. (A) Surface expression of CD300a on mast cells, monocytes freshly isolated from PBMCs, or MDMs was monitored by flow cytometry. Gray shading represents cell staining with control Ab. (B) Cells were infected with DENV2 JAM (MOI = 5) and, 24 h later, infection was assessed by flow cytometry with the 2H2 MAb. (C) Surface expression of CD14 and known DENV receptors on MDM were monitored by flow cytometry using respective antibodies and control isotypes. MDMs were preincubated for 30 min with goat anti-CD206, anti-CD300a, anti-TIM4, or goat IgG as a control prior to infection with DENV2-JAM (MOI = 5) in the continuous presence of the polyclonal Ab. The percentages of infected cells were assessed 24 h postinfection by flow cytometry and normalized to infection in the presence of control IgG. The data shown are means ± the SD from experiments performed in duplicate on MDM from three independent donors. (ns nonsignificant; **, $P < 0.001$).

not inhibit the binding of DENV to receptors specific of PtdSer such as mo CD300a or TIM-3 (Fig. 5E). Altogether, these results strongly suggest that hu CD300a interaction with DENV largely relies on the recognition of virion-associated PtdEth, whereas mo CD300a interaction with viral particle essentially depends on virion-associated PtdSer.

The IgV-like domains of CD300a and TIM receptors present similar structures (29). The D106 and D115 residues are required for binding of CD300a to PtdEth and PtdSer and line a cavity where the hydrophilic head of the phospholipids is thought to penetrate (29). To investigate the role of these residues in DENV-CD300a interactions, we generated HEK293T stably expressing human CD300a mutants D106A or D115A and tested their ability to promote infection. The two mutants were correctly expressed at the cell surface (Fig. 6A) but did not enhance DENV infection (Fig. 6B), indicating that the "MILIBS-like" pocket in CD300a IgV domain is essential for the enhancement of DENV infection.

Anti-CD300a antibodies block DENV infection of human MDMs. We next investigated the role of CD300a in human primary cells. Flow cytometry analyses showed that CD300a is expressed at the surface of mast cells, monocytes, and MDMs (Fig. 7A). We did not detect CD300a expression in other cells, such as B or T cells (data not shown). Mast cells, monocytes, and MDMs were infected with the DENV2 JAM strain, and viral infection was evaluated by FACS using 2H2 MAb. As shown in Fig. 7, MDM were productively infected by DENV, whereas monocytes and mastocytes were insensitive. Therefore, CD300a expression by itself is not sufficient to confer sensitivity to DENV, suggesting a cell type-specific role of CD300a during DENV infection. In addition to CD300a, macrophages express the mannose receptor (CD206)

and low levels of TIM-4 (Fig. 7C). Other surface receptors known to play a role in DENV entry (TIM-1, Axl, and DC-SIGN) were not detected (Fig. 7C). To evaluate the role played by these molecules in DENV infection of MDMs, we performed infection studies in the presence of anti-CD300a, TIM-4, or CD206 neutralizing Abs (9, 12). The anti-CD300a partially but significantly blocked DENV infection of MDMs, whereas anti-CD206 or anti-TIM-4 Ab had no effect.

Conclusions. We identified CD300a as a new phospholipid receptor that directly binds to DENV particles and enhances infection. In contrast to TIM and TAM receptors, which mediate infection by recognizing specifically virion-associated PtdSer, CD300a-dependent infection relies essentially on the recognition of PtdEth and to a lesser extent PtdSer on the surface of viral particles. PtdEth has been detected, along with PtdSer, on the luminal leaflet of the endoplasmic reticulum (ER) membrane, the site of virus budding (32). Thus, the ER-derived membrane of DENV may incorporate both PtdSer and PtdEth. CD300a enhances infection of several members of the flavivirus genus, including the four DENV serotypes, WNV, and YFV, as well as the Chikungunya alphavirus. It has been recently reported that CD300a binds lentiviral vector particles pseudotyped with Sindbis virus envelope (2.2 1L1) but does not mediate transduction (33). It is possible that the relative levels of exposed PtdEth may differ between DENV particles and pseudotyped lentiviral vectors. We showed that ectopic expression of CD300a enhanced viral internalization by clathrin-mediated endocytosis. This observation, together with the fact that CD300a expression mediates DENV infection in the absence of its cytoplasmic tail, suggests that CD300a does not act as a bona fide receptor for flaviviruses but rather as an attachment factor that transfers viral particles to other molecules. In addition, DENV binding to CD300a-expressing cells could induce signals regulating a postentry step of virus infection. Indeed, CD300a is a signaling molecule that is activated upon apoptotic cell binding and recruits the phosphatase SHP-1 to inhibit release of lipopolysaccharide (LPS)-induced inflammatory cytokines in murine mast cells (30). Similar inhibition of TLR-dependent inflammatory pathways involved in the clearance of invading pathogens has been obtained by activating endogenous CD300a in monocytic cell lines exposed to LPS or CpG pathogen-associated microbial patterns (34). Further studies are required to investigate whether the ligation of CD300a by DENV may modulate innate immune responses for optimal infection.

In conclusion, our results show that CD300a is a novel DENV binding receptor that recognize mainly PtdEth present on the surface, of virions, reinforcing the concept that DENV uses an apoptotic mimicry strategy for productive entry.

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